Bioorganic & Medicinal Chemistry 18 (2010) 8026–8034

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Naphthylchalcones induce apoptosis and caspase activation in a leukemia cell line: The relationship between mitochondrial damage, oxidative stress, and cell death

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ARTICLE INFO

Article history:
Received 9 July 2010
Revised 3 September 2010
Accepted 9 September 2010
Available online 16 September 2010

Keywords:
Leukemia
L1210 cells
Chalcones
Apoptosis
Oxidative stress

ABSTRACT

In this study, we investigated the effects of 24 chalcone derivatives from 2-naphthylacetophenone toward a lymphoblastic leukemia cell line (L1210). Three compounds, called **R7**, **R13**, and **R15**, presented concentration- and time-dependent cytotoxicity and induced cellular death by apoptosis via mitochondrial injury and oxidative stress. The effects of these compounds appear to occur through different mechanisms because **R13** and **R7** induced a greater disturbance of mitochondrial potential, and all compounds induced disturbances of cellular ATP content and increased caspase-3 activity before cellular death. These compounds also interfered with antioxidant enzymes activities and GSH content through different mechanisms.

1. Introduction

Acute lymphoblastic leukemia (ALL) is a malignant disorder of lymphoid progenitor cells that affects both children and adults, with peak prevalence between the ages of two and five years.[1] Although contemporary treatments cure more than 80% of children with ALL,[2] some patients require intensive treatment, and many patients still develop serious acute and late complications due to the side effects of the drugs. Furthermore, the survival rate for adults with ALL remains below 40%,[3] and multidrug resistance is frequently reported.[2] Therefore, new drugs and treatment strategies are needed to improve both the cure rate and patient quality of life.[4] Studies have considered that due the heterogeneity of the alterations found in leukemias, a combination therapy with antiproliferatives and apoptosis-inductive agents could be more effective than conventional treatments.[5]

Cell death through apoptosis can result from several cellular events, occasioned frequently by mitochondrial alterations and the consequent alterations in oxidative metabolism and cellular content of ATP.[6] The process of apoptosis leads to characteristic changes in cells such as DNA fragmentation, chromatin condensation, cell shrinkage, and membrane blebbing.[7]

Several recent studies have shown that the use of drugs that compromise the structural and functional integrity of mitochondria can be an alternative treatment against the growth of neoplastic cells.[8] When a death signal is detected by the mitochondria, a collapse of mitochondrial potential is triggered, and apoptotic factors as cytochrome *c* are released to the cytosol. These factors activate caspases, culminating in apoptosis.[9] Alterations in mitochondria can also lead to bioenergetic imbalances because these organelles are responsible for the production of 80 to 90% of the ATP required by cells.[8]

The mechanisms of cellular death also can be influenced by an imbalance in oxidative metabolism called oxidative stress. The mitochondrion is the main organelle involved in this phenomenon because it is the primary consumer of cellular oxygen and the center of reactive oxygen species (ROS) generation.[6] Under oxidative stress, normal cells can enter into an adaptation process, mainly through their antioxidant defenses.[10] However, tumor cells can have a limited capacity of adaptation for some defenses; therefore, antitumor agents that induce the production of ROS have potential therapeutic benefits.[11] Studies have related the increase of ROS with the increase of cellular death.[12]

Chalcones are essential intermediate compounds in flavonoid biosynthesis in plants. Many studies have demonstrated antitumor,

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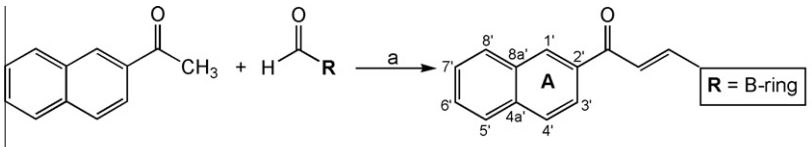
doi:10.1016/j.bmc.2010.09.025



R7, R = 3-NO_2-phenyl
R8, R = 4-NO_2-phenyl
R10, R = 4-Cl-phenyl
***R11**, R = 3,4-Cl_2-phenyl
R12, R = 2,6-Cl_2-phenyl
R13, R = 3-Cl-phenyl
R14, R = 2-Cl-phenyl
R15, R = phenyl
R16, R = 4-CH_3-phenyl
R17, R = 4-OCH_3-phenyl
R19, R = 2,6-OCH_3-phenyl
R20, R = 2,4,5-OCH_3-phenyl
R21, R = 4-$N(CH_3)_2$-phenyl
***R23**, R = 4-$O(CH_2)_3CH_3$-phenyl
R24, R = 4-F-phenyl
***R25**, R = 2-F-phenyl
R26, R = 4-Br-phenyl
R27, R = 3,4-OCH_2O-phenyl
R28, R = 2-naphthyl
R29, R = 1-naphthyl
***R44**, R = 2,4,6-OCH_3-phenyl
***R45**, R = (3-OCH_3-4-OCH_2-phenyl)-phenyl
R30, R =
R36, R =

Figure 1. Preparation of chalcones. (a) KOH 50% m/v, methanol, rt, 24 h. *Novel compounds.

anti-inflammatory, anti-infective, and hypotensive activities for chalcones, in addition to other pharmacological effects (for reviews, see Refs. 13–15). Kachadourian and Day[16] demonstrated that the antileukemic activity of hydroxychalcones occurred via depletion of reduced glutathione (GSH), release of cytochrome *c* and loss of mitochondrial membrane potential. Navarini et al.[17] also demonstrated that the antileukemic activity of hydroxychalcones occurred via depletion of GSH and ATP.

Despite advances in the therapies, the greatest problem associated with acute leukemia is the resistance to systemic methods of treatment that lays the importance of investigation of new potential molecules to control and/or cure this disease. In this study, we investigated the effects of chalcone derivatives from 2-naphthylacetophenone toward a lymphoblastic leukemia cell line by characterizing cell death and by monitoring induced oxidative stress and the influence of the derivatives on energy metabolism, never studied before in this regard.

2. Results and discussion

2.1. Chalcone synthesis

Twenty-four chalcones were prepared in methanol under basic conditions by aldolic condensation between 2-naphthylacetophenone and corresponding aldehydes (Fig. 1),[18] providing compounds with substituents on the B-ring. The obtained yields varied between 29% and 97%. All reagents used were obtained commercially (Sigma–Aldrich), except the benzylated vanillin [3-methoxy-4-(phenylmethoxy)-benzaldehyde], which was prepared as previously described,[19] with a yield of 88%. All structures, including those not yet published (**R11**, **R23**, **R25**, **R44**, and **R45**), were confirmed by melting points and by chemical identification data: infrared spectroscopy (IR), ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR), and elementary analysis. ^{1}H NMR spectra revealed that all structures were structurally pure and with *E* configuration ($J_{H\alpha-H\beta}$ = 16.0 Hz).

2.2. Biological

To screen and compare the cytotoxicity induced by the naphthylchalcones, all compounds were incubated in the L1210 cell line at 25, 50, 75 or 100 μM for 24 h and the IC_{50} values were estimated. Chalcones **R7**, **R13**, **R14**, **R15**, **R25**, and **R30** were the compounds that presented the best cytotoxic effect, with IC_{50} values below 40 μM (Fig. 2). It is important to observe that these compounds are structures either without substituents on the B-ring (**R15**), with substituents that reduce the electron density of the aromatic ring in positions 2 or 3 of the B-ring (**R7**, **R13**, **R14**, and **R25**) or with a sulfur heterocyclic as the B-ring (**R30**). Chalcones with substitutions at position 3, accompanied by another substitution at position 4 of the B-ring, showed moderated activity (**R11** and **R27**), as well as **R12** (2,6-dichlorated), **R29** (with a 1-naphthyl

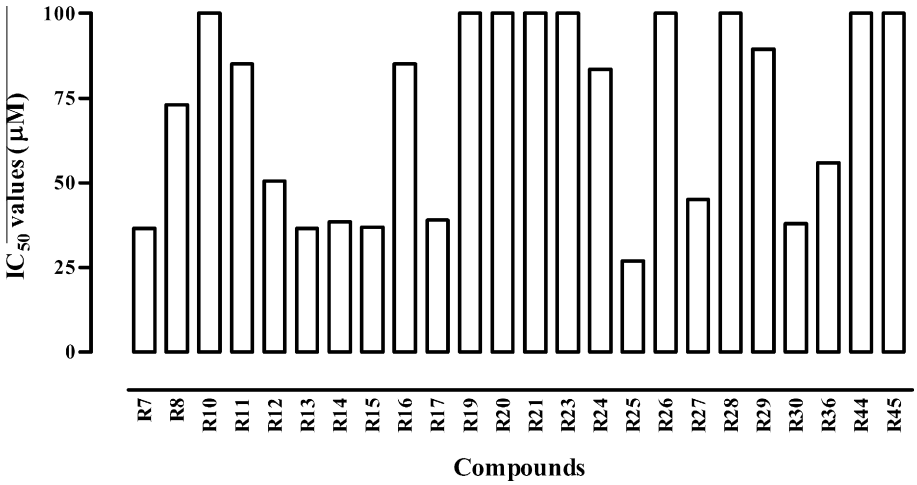


Figure 2. Cytotoxicity of naphthylchalcones in L1210 cells. Cells were incubated with the compounds at concentrations of 25, 50, 75, and 100 μM at 24 h, and cell viability was assayed by MTT. Optical density of zero time was taken as 100% cell viability.

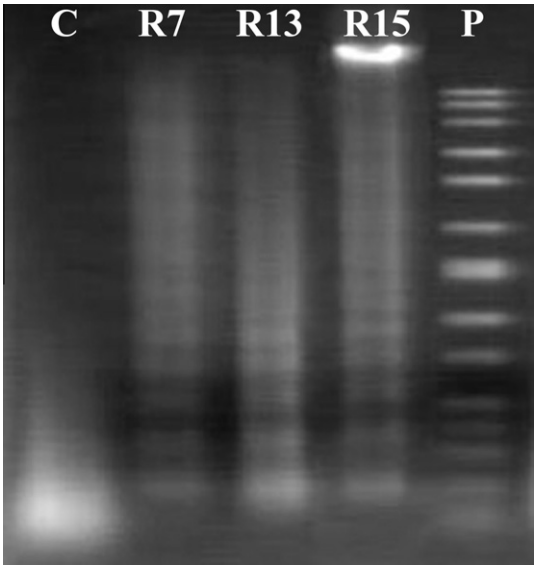


Figure 3. DNA fragmentation in L1210 cells induced by chalcones **R7**, **R13**, and **R15**. Cells were incubated with the compounds for 24 h. DNA analysis was performed through agarose gel electrophoresis. C represents the control cells (cells incubated without chalcones), and P represents the DNA sizing standard. **R7** 23 μM, **R13** 37 μM, **R15** 36 μM.

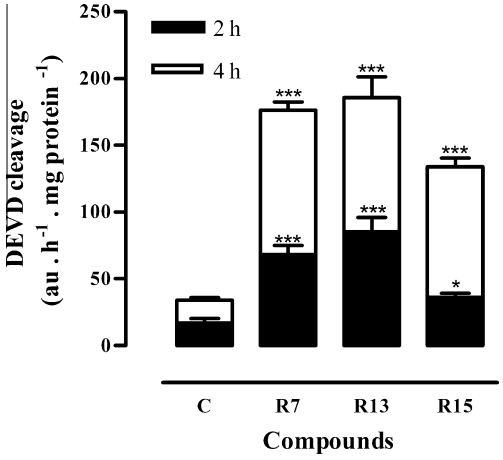


Figure 4. Activation of caspase-3 activity by chalcones **R7**, **R13**, and **R15** in L1210 cells. Cells were incubated with the compounds for 2 and 4 h. Caspase-3 activity was measured by monitoring the cleavage of Ac-DEVD-AMC, a fluorogenic substrate for caspase-3, for 2 h. The activity is given in arbitrary fluorescence units (a.u.) per hour per μg protein. $^{*}p$ <0.05 and $^{***}p$ <0.001. **R7** 23 μM, **R13** 37 μM, **R15** 36 μM.

group as B-ring) and **R36** (with an oxygenated heterocyclic ring). Mono-substituted compounds with substitutions at position 4, or compounds substituted at position 4 and one substitution more at available positions, presented moderated (**R8**, **R16**, **R17**, and **R24**) or no activity (**R10**, **R20**, **R21**, **R23**, **R26**, **R44**, and **R45**). These results indicate that the substitutions at position 4 of the B-ring decrease the cytotoxic potential of chalcones against L1210 leukemia cells when the A-ring is a 2-naphthyl group. These observations are consistent with Nam et al.[20] who observed that chalcones with electron-withdrawing groups at the B-ring, mainly at 2-position, confer strong angiogenesis inhibition and marked antitumor activity in murine melanoma (B16), human colon cancer (HCT116), and human epidermoid carcinoma cells (A431).[20] In addition, it was shown that compounds **R28** (with a 2-naphthyl group as B-ring) and **R19** (dimethoxylated in positions 2 and 6 of B-ring) presented no cytotoxic activity.

Chalcones **R7**, **R13**, **R14**, **R15**, **R25**, and **R30** were selected and analyzed for induction of cell death. Compounds **R7**, **R13**, and **R15** induced cell death by apoptosis, characterized by internucleosomal breakdown of DNA (Fig. 3). **R7**, **R13**, and **R15** demonstrated cytotoxicity to L1210 and the IC_{50} values are shown in the Table 1. These chalcones were also cytotoxic to a human leukemia cell line (CEM) with lower values of IC_{50} (Table 1). The cell death mechanism for this cell line was also by apoptosis, observed by DNA breakdown pattern (data not shown). As can be observed in the Table 1 the values of IC_{50} in function of time decreased for both cell lines, principally after 72 h of incubation with the compounds, with the exception of R15 for L1210. These results, although not conclusive, indicate that the chalcones **R7**, R13, and **R15** may present also antiproliferative activity in vitro.

Table 1
Comparison between the IC_{50} values of the chalcones for cell viability in L1210 and CEM cell lines

Compounds	L1210 IC_{50} (μM)			CEM IC_{50} (μM)		
	24 h	48 h	72 h	24 h	48 h	72 h
R7	24	13	14	11	11	4
R13	38	23	10	11	12	6
R15	37	20	17	12	14	5

Several studies have searched for new drugs with the ability to induce apoptosis in leukemic cells.[4,21] To confirm whether or cell death was induced by apoptosis, cells were incubated with chalcones **R7**, **R13**, and **R15** at their respective IC_{50} concentrations (**R7** 23 μM, **R13** 37 μM, **R15** 36 μM) for 2 and 4 h to determine caspase-3 activity. The compounds increased caspase-3 activity (Fig. 4), confirming that chalcones **R7**, **R13**, and **R15** induced cell death by apoptosis. In a previous study with the HL-60 cell line, Nakatani et al.[22] showed the antileukemic activity of dihydrochalcones by caspase activation. However, these chalcones differ from ours by not having a double bond.

To determine whether oxidative stress was involved in chalcone-induced cell death, the generation of ROS was evaluated by the extent of lipid peroxidation following a concomitant incubation of chalcones (**R7** 23 μM, **R13** 37 μM, **R15** 36 μM) and antioxidant enzymes. Cell viability was evaluated in parallel. As shown in Figure 5A, all compounds significantly induced ROS production, and this production was blocked when catalase (CAT) was present. Co-incubation of the compounds with catalase also increased cell viability after 24 h of incubation when compared with compounds as single agents. These differences were not observed when compounds were co-incubated with superoxide dismutase (SOD) or Trolox® (TR). These results are summarized in Table 2, including the IC_{50} values obtained with these treatments. Additionally, all chalcones induced lipid peroxidation of cell membranes (Fig. 5B). Therefore, these findings indicate that the compounds tested induced oxidative stress and suggest that hydrogen peroxide (H_2O_2) can be the main ROS involved in the process because the treatment of the cells with catalase decreased the production of ROS and the resulting cytotoxicity.

Many natural compounds can induce apoptotic cell death in cancer cells by increasing ROS content, promoting the formation of pores in mitochondria.[8] The relation between ROS mitochondrial production, cytochrome *c* release and caspase-3 activation in a leukemic cell line was also reported.[23] Jing et al.[24] and Shin et al.[12] demonstrated the antileukemic activity of different compounds by induction of apoptosis via ROS generation, which resulted in a decrease in mitochondrial membrane potential. Thus, we evaluated the effect of chalcones (**R7** 23 μM, **R13** 37 μM, **R15** 36 μM) on mitochondrial membrane potential and observed that compounds **R7** and **R13** decreased mitochondrial membrane potential (Fig. 6D) to a similar extent as did carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). These findings can be related

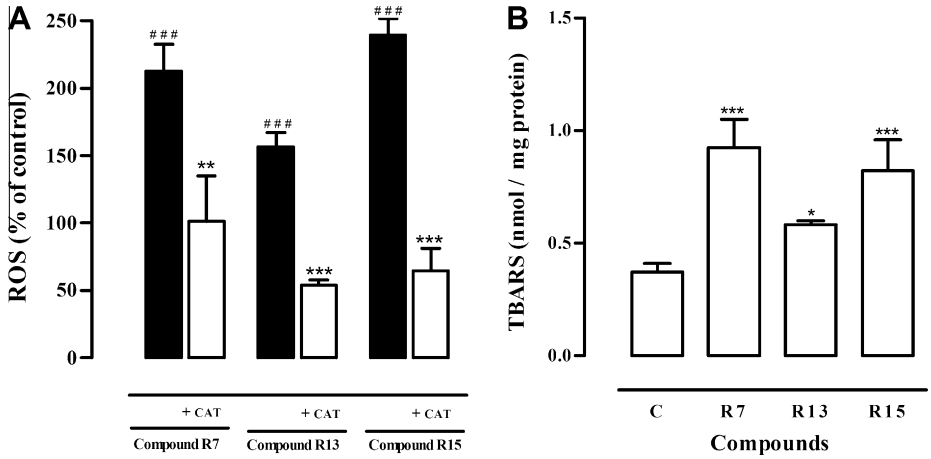


Figure 5. Effects of chalcones **R7**, **R13**, and **R15** on ROS generation and lipid peroxidation in L1210 cells. (A) ROS formation was followed using DCFH-DA. The results are expressed as the percentage of cellular fluorescence in comparison to control samples (zero % of fluorescence). ###p <0.001 when the control samples and the cells incubated with chalcones alone were compared, and **p <0.01; ***p <0.001 when the cells incubated with chalcones alone and the cells incubated with chalcones plus catalase were compared. (B) Lipid peroxidation was measured using the TBARS method. The results are expressed as nmol of TBARS per μg of protein. *p <0.05 and ***p <0.001. **R7** 23 μM, **R13** 37 μM, **R15** 36 μM.

Table 2
Comparison between the IC_{50} values for cytotoxicity of the chalcones **R7**, **R13**, and **R15** incubated alone and associated to catalase, Trolox®, and superoxide dismutase with L1210 cell line

Treatments	IC_{50} (μM)
R7	24 ± 0.6
R7 + CAT	45 ± 1.8***
R7 + SOD	20 ± 0.5
R7 + TR	20 ± 1.0
R13	38 ± 1.2
R13 + CAT	51 ± 2.4**
R13 + SOD	30 ± 0.3
R13 + TR	34 ± 1.9
R15	37 ± 1.1
R15 + CAT	58 ± 2.8***
R15 + SOD	31 ± 0.7
R15 + TR	39 ± 0.5

** p <0.01.
*** p <0.001

with ROS generation caused by the compounds and consequently with oxidative stress. Sastre et al.[25] reported that ROS can cause oxidative damage to protein, lipids and respiratory chain proteins activating caspase and other apoptotic factors. Chalcone **R15** did not significantly change mitochondrial membrane potential (Fig. 6D), although it was the compound that induced the highest level of ROS generation. This increase can be related with its potential to induce ROS generation in distinct cell site not in mitochondria.

The mitochondria are the central organelles involved in apoptosis and are responsible for ATP production in cells. Therefore, the determination of the intracellular ATP concentration is important in researching drugs that induce apoptosis and deplete energy.[26] Chalcones **R7**, **R13**, and (**R7** 23 μM, **R13** 37 μM, **R15** 36 μM) induced a slight variation of ATP concentration, decreasing and increasing at initial times of measurements and finally a strong ATP concentration depletion (Fig. 6A–C). It is important to mention that the ATP measured was from the remaining live cells. Sabzevari et al.[27] showed that the cytotoxic effects of 10 hydroxychalcones in a leukemic cell line resulted from mitochondrial disturbances. In this study, similar effects occurred, resulting in respiratory chain deregulation and consequently depletion of ATP content and increased ROS production. Increased ROS levels can activate JNK and p38 and induce cell death. Skulachev[28] evaluated the relationship between apoptosis and ATP content and showed that mitochondrial damage can result in a decrease of ATP content and apoptosis, or an initial increase in ATP content followed by a decrease in ATP content and cell death. The results of Skulachev may help to explain the increase of ATP content that occurred in cells incubated with chalcones observed in the current study. As shown in Fig. 6A and B, chalcones **R7** and **R13** decreased mitochondrial membrane potential and consequently caused damage in the respiratory chain and depletion of ATP content. An alternative mechanism occurred with chalcone **R15** (Fig. 6C). The direct depletion of ATP can be related with the strong pro-oxidant effects of this chalcone, as observed in Figure 5A, and with Glutathione peroxidase (GPx) and Glutathine *S*-transferase (GST) activities in Figure 7. Cao et al.[29] reported that high doses of curcumin induced damage to mitochondrial DNA due to the increase in ROS. mtDNA damage decreases the operation of the respiratory chain, and consequently, it strongly decreases ATP synthesis.

To minimize the damage of ROS, aerobics organisms were endowed with enzymatic and not-enzymatic antioxidant defenses. The principal enzymatic defenses are catalase (CAT), glutathione reductase (GR), GPx, and GST.[30] GST do not act directly against ROS, but they are important to cellular defenses as part of the phase II detoxification enzyme family that is responsible for the protection of cellular macromolecules from attack by reactive electrophiles via GSH conjugation.[31] Among the non-enzymatic antioxidants are mainly melanin, glutathione, uric acid, vitamins C and E and lipoic acid.[30] Chalcones **R7**, **R13**, and **R15** significantly increased the activity of GPx (Fig. 7A), GST (Fig. 7B) and GR (Fig. 7C), but none of them affect CAT activity (Fig. 7D). All chalcones increased glutathione oxidized (GSSG) (Fig. 8A) and total glutathione (TG) (Fig. 8B) contents. Chalcones **R7** and **R13** did not change GSH content, but **R15** increased GSH content (Fig. 8C) The concentration of the chalcones used was **R7** 23 μM, **R13** 37 μM, **R15** 36 μM.

The increase in GPx activity can be explained by the increases in ROS production and lipid peroxidation induced by the chalcones. Because GPx is responsible for the reduction of peroxides, an increase in peroxide formation can result in an increase of GPx activity. The increase in GST occurred perhaps because these chalcones possibly are eliminated by conjugation with GSH by GST. Thus, an increase in chalcones concentration can result in GST activity enhancement. Surprisingly, the chalcones augmented GPx activity but did not increase CAT activity even though CAT is the principal enzyme responsible for reducing H_2O_2. On the contrary, chalcones appears

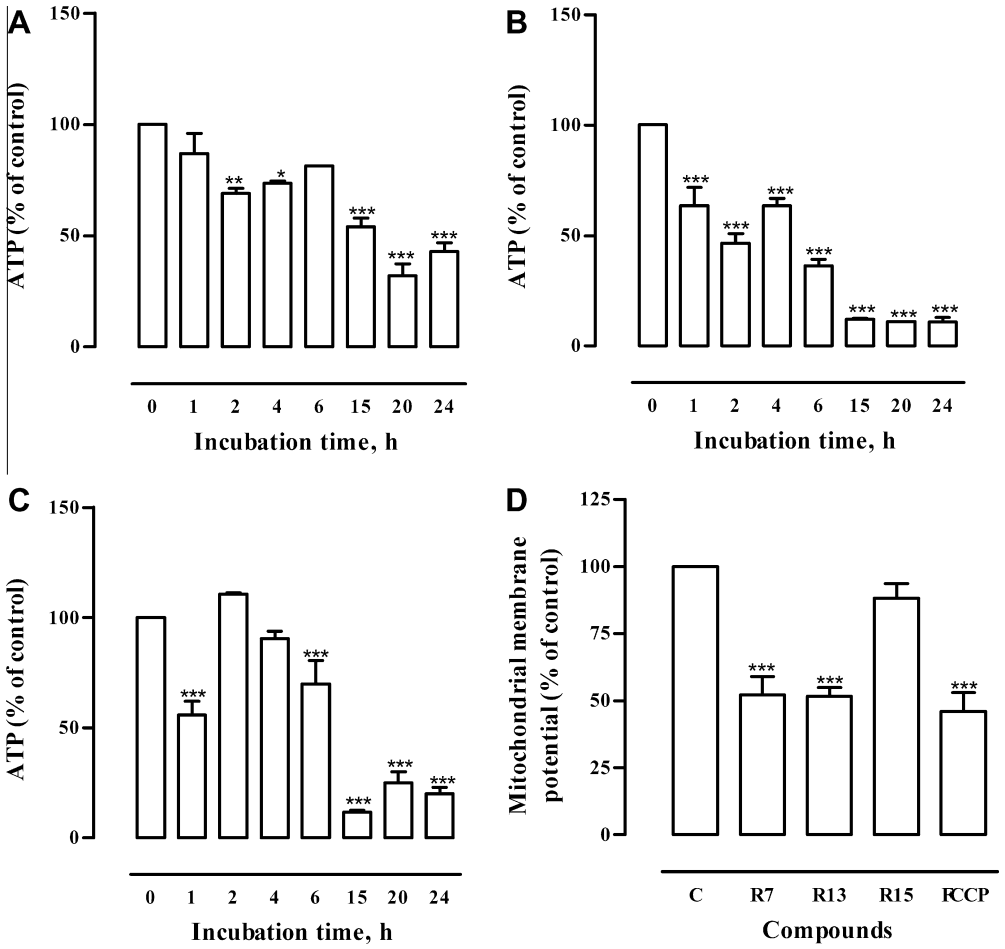


Figure 6. Effect of chalcones **R7**, **R13**, and **R15** on mitochondrial membrane potential and intracellular ATP content in L1210 cells. ATP concentration was monitored by the luciferin–luciferase assay following incubation with chalcones at **R7** 23 μM, **R13** 37 μM, **R15** 36 μM for different incubation times: (A) **R7**, (B) **R13**, and (C) **R15**. (D) The membrane potential was determined using JC-1 after 4 h of incubation. A decrease in the red/green ratio indicates a decrease in mitochondrial membrane potential. The results are expressed as percentages and the values of control cells were considered 100%. ***p <0.01; **p <0.01; ***p <0.001.

to inhibit CAT activity. We investigated this hypothesis by evaluating the effects of chalcones on the activity of isolated CAT enzyme. Although the test was performed with an enzyme batch isolated from a different source, chalcones, principally **R13**, significantly inhibited CAT activity (data not shown). Pelicano et al.[11] described that cancer cells can have a limited capacity of adaptation for some antioxidant defenses in response to oxidative stress. The inability of cells to increase CAT activity, in this case, can be the reason of the cytotoxicity of chalcones because it is related mainly to the generation of H_2O_2. Another important fact resulting from the action of ROS in cancer cells is the oxidation of proteins such as cytochrome *c* and CAT. CAT inhibition can reduce the capacity of cells to eliminate H_2O_2, increasing cellular damage induced by ROS.[32]

GSH is the most important non-enzymatic antioxidant. Normally, the majority of GSH remains reduced by GR. However, oxidative stress results in a decrease in GSH content and consequently, to an increase in GSSG content.[33] Chalcones **R7**, **R13**, and **R15** induced an increase in GSSG content, more evidence that these compounds induce oxidative stress. The increase in GR can be the result of the oxidative stress occurring during the incubation of cells with chalcones in an attempt to sustain GSH content. This result also explains why GSH content decreased in cells during incubation with chalcones **R7** and **R13** and why GSH content increased during incubation with chalcone **R15**. Moreover, the observed increase in GSH content can be the result increased γ-glutamyl-cysteine-synthetase (γ-GCS) activity promoted by chalcone **R15** (data not shown).

3. Conclusion

The development of new drugs for the treatment of cancer is based on the potential of compounds to block cell proliferation and induce apoptosis.[34] Our findings showed, for the first time, that the naphthylchalcones **R7**, **R13**, and **R15** present cytotoxicity inducing apoptosis in leukemia cell line L1210 through distinct targets. In general, these compounds induced oxidative stress; however, for chalcone **R7** and **R13**, the stress is a consequence of mitochondrial damage that can be related with respiratory chain damages and the deregulation of ROS generation and subsequent depletion of ATP content. For chalcone **R15**, cellular death results from its strong pro-oxidant effect that was induced mainly by ROS generation and interference with antioxidant defenses. Our group targeting if the chalcones have antiproliferative effect behind cell cycle cell studies is developing further studies with both cell lines.

These results indicate that these compounds are interesting in the research of new drugs for cancer treatment.

4. Experimental part

4.1. Chemicals

The cell culture media were purchased from Cultilab (São Paulo, Brazil). Serum and antibiotics were purchased from GIBCO (Grand

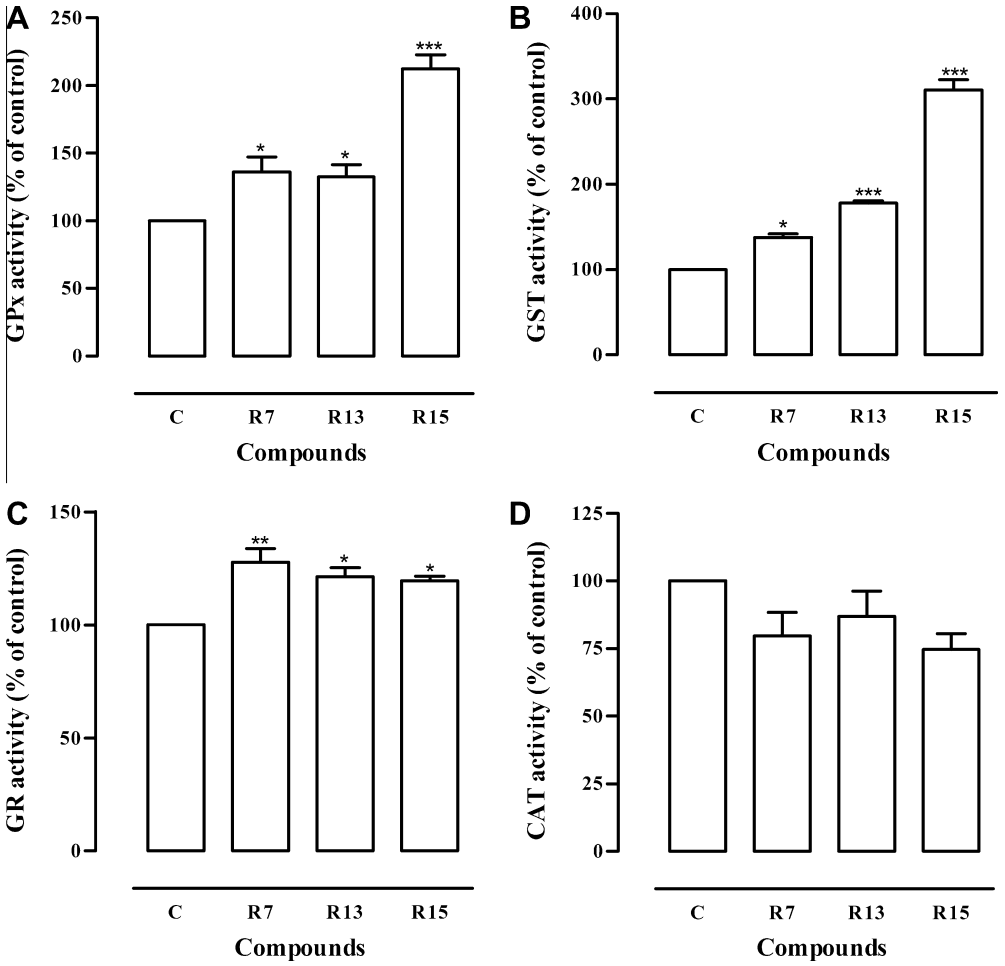


Figure 7. Effect of chalcones **R7**, **R13**, and **R15** on antioxidant defenses. Activities of GPx (A), GST (B), GR (C), and CAT (D) were measured as described in Section 4 and presented as percentages of control. **R7** 23 μM, **R13** 37 μM, **R15** 36 μM. $^{*}p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001. The incubation time was 24 h.

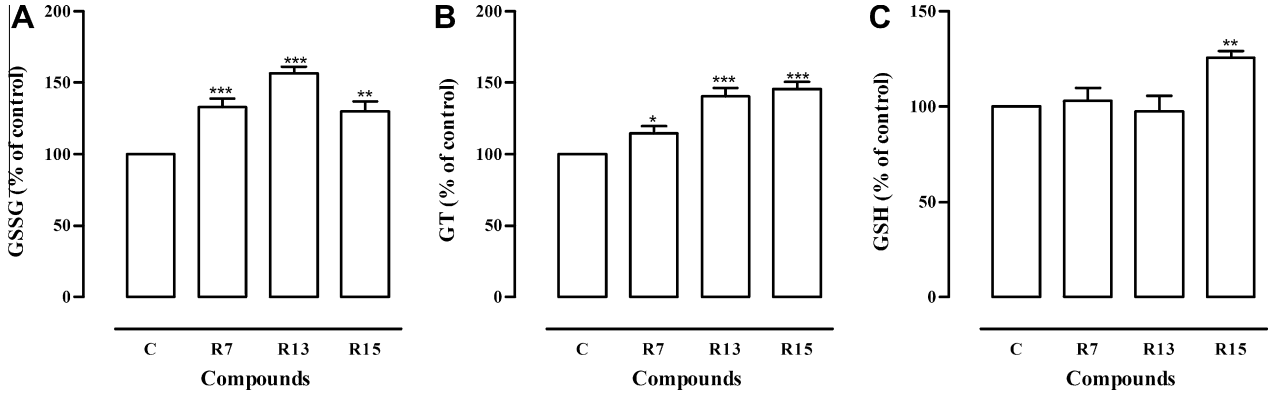


Figure 8. Effect of chalcones **R7**, **R13**, and **R15** on GSH, GSSG, and TG content. Content of GSSG (A), TG (B), and GSH (C) were measured as described in Section 4 and presented as percentages of control. **R7** 23 μM, **R13** 37 μM, **R15** 36 μM. $^{*}p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001. The incubation time was 24 h.

Island, NY). The luciferin–luciferase kit was purchased from Bioorbit (Tuku, Finland), the JC-1 was purchased from Invitrogen and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

4.2. Preparation of compounds

Reagents used were obtained commercially (Sigma–Aldrich®), except the benzylated vanillin [3-methoxy-4-(phenylmethoxy)-benzaldehyde], which was prepared as previously described.[19] All chalcones were prepared by aldolic condensation between 2-naphthylacetophenone and corresponding aldehydes, in methanol, KOH (50% v/v), at room temperature with magnetic agitation for 24 h.[18] Distilled water and 10% hydrochloric acid were added to the reaction for total precipitation of the compounds, which were then obtained by vacuum filtration and later recrystallized in dichloromethane and hexane. The chalcones are soluble in dimethylsulfoxide, acetone, chloroform and dichloromethane. Chalcones

R7, **R8**, **R10**, **R12**, **R13**, **R14**, **R15**, **R16**, **R17**, **R20**, **R21**, **R24**, **R26**, **R27**, **R28**, **R29**, and **R30** were previously cited in the literature (see Refs. 35–41). **R19** and **R36** were recently published by our group,[42] and **R11**, **R23**, **R25**, **R44**, and **R45** are new compounds.

4.3. Physico-chemical data of the compounds

The structures were confirmed by melting points (mp), infrared spectroscopy (IR), and ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR), as well as elementary analysis for previously undescribed structures. Melting points were determined with a Microquímica MGAPF-301 apparatus and are uncorrected. IR spectra were recorded with an Abb Bomen FTLA 2000 spectrometer on KBr disks. NMR (^{1}H and ^{13}C) spectra were recorded on a Varian Oxford AS-400 (400 MHz) instrument, using tetramethylsilane as an internal standard. Elementary analysis was carried out using a CHNS EA 1110; percentages of C and H were in agreement with the product formula (within ±0.4% of theoretical values for C).

4.4. Cell culture

Murine lymphoblastic leukemia (L1210) and human lymphoblastic leukemia cells (CEM) were obtained from American Type Culture Cell (ATCC). The cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM HEPES. The cell culture was maintained at 37 °C in a 5% CO_2 humidified atmosphere and pH 7.4. Every 2–3 days, cells were passaged by removing 90% of the supernatant and replacing it with fresh medium. In all experiments, viable cells were checked in the beginning of the experiment by Trypan Blue exclusion.

4.5. Cytotoxicity

Chalcone cytotoxicity was evaluated by MTT assay.[43] To evaluate the influence of concentration on cytotoxicity, 1×10^5 and 5×10^4 cells/well were incubated for 24 and 48 h, respectively, in triplicate with the compounds (solubilized in DMSO no more than 1%), and at different concentrations (1–100 μM) in 96-well microplates. To evaluate the cytotoxicity of chalcones in combination with catalase, 1×10^5 cells/well were incubated for 24 h with different concentrations (1–100 μM) of the compounds and plus CAT (5000 U/ml), SOD (250 U/ml) and TR (200 μM) a water-soluble vitamin E derivative. To verify if the enzymes and TR could themselves to prejudice the cell, a control with the same additions without the chalcones was run in parallel; cell viability remained without alterations. After incubation at 37 °C, cells were washed with fresh culture medium, and 10 μl of MTT (5 mg/ml) were added followed by 2 h incubation at 37 °C. The precipitated formazan was dissolved in 100 μl of DMSO, and the absorbance was measured at 540 nm using a microwell system reader. The IC_{50} values (a concentration that produces 50% reduction in of the viable cell number) were calculated through a Hill concentration–response curve. Cell viability was checked in the beginning of the experiment by Trypan Blue exclusion. The chalcones were dissolved in DMSO, and to verify if the solvent itself could affect the cells, in all experiments, control curves without chalcones and in the presence of the cells and the solvent were carried out in parallel. The controls with solvent were not statistically different from control cells alone.

4.6. Analysis of DNA fragmentation

The isolation of apoptotic DNA fragments was based on the method of Han.[44] Briefly, 3×10^6 L1210 cells were incubated with the compounds for 24 h (at the IC_{50} concentration for each compound). Cells were then washed with cold PBS and incubated with lysis buffer (10 mM EDTA, 50 mM Tris–HCl pH 8.0, 0.25% NP-40, 0.5 g/L proteinase K) at 50 °C for 2 h. DNA was then precipitated with 2.5 vol of ethanol at 25 °C overnight and dried in air. After washing with ice-cold 70% ethanol, the pellets were dissolved in TE buffer containing 10 mM Tris–HCl pH 8.0, 1 mM EDTA, and 0.6 g/ml RNase A, and they were further incubated at 37 °C for 1 h. Horizontal electrophoresis was performed at 150 V using a 1.0% agarose gel with TAE (Tris–acetic acid and EDTA) as the running buffer. The gel was stained with ethidium bromide and visualized by a 2UV Transilluminator (MacroVue UV-20 Hoefer) for ladder formation.

4.7. Determination of caspase-3 activity

To determine the activity of caspase-3, 3×10^6 L1210 cells were incubated with the compounds for 2 and 4 h (at the IC_{50} concentration for each compound) at 37 °C. The cells were then washed with PBS and lysed in 75 μl of lysis buffer containing 10 mM HEPES pH 7.4, 42 mM KCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonylfluoride (PMSF), 0.1 mM EDTA, 0.1 mM EGTA, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 5 μg/ml aprotinin, 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), and 1 mM dithiothreitol (DTT) at 4–8 °C for 5 min. The extract (50 μg of protein) was then added to a buffer containing 25 mM HEPES pH 7.4, 0.1% CHAPS, 1 mM EDTA, 10% sucrose, and 3 mM DTT. The reaction medium was supplemented with 10 μM Ac-DEVD-AMC caspase-3 substrate (acetyl-Asp-Glu-Val-Asp-amino-methylcoumarin), a fluorogenic substrate for caspase-3. After incubation at 37 °C for 2 h, caspase activity (production of fluorescent AMC) was monitored using a spectrofluorimeter (Perkin–Elmer LS55) measuring the extinction at 380 nm and emission at 465 nm. The fluorescence of blanks containing no cellular extracts was subtracted from the fluorescence sample values.[45] Protein content was determined by Lowry's method[46] and caspase-3 activity was expressed as a change in fluorescent units per hour per μg of protein.

4.8. Free radical determination

Intracellular free radical formation was determined using 2′,7′-dichlorofluorescein diacetate (DCFH-DA), which is oxidized to dichlorofluorescein (DCF) in the presence of ROS.[47] 5×10^5 L1210 cells were incubated with the compounds (at the IC_{50} concentration of 24h for each compound) in the absence or presence of 5000 U/ml catalase for 4 h at 37 °C. Cells were then incubated with 10 μM DCFH-DA for 30 min at 37 °C and then washed four times with PBS. The DCF fluorescence signal was measured using a Perkin–Elmer LS55 spectrofluorimeter. The results were normalized by the cell death percentage that occurs during 4 h of incubation.

4.9. Preparation of homogenates

Cell homogenates were prepared to complete the lipid peroxidation, glutathione and enzyme assays. For enzyme assays, 6×10^6 cells were used, and for glutathione and lipid peroxidation measurements, 3×10^6 cells and 4×10^6 cells were used, respectively. These cells were incubated with the compounds at the IC_{50} concentration for each compound for 24 h. The samples were washed twice with PBS, lysed with 20 mM phosphate buffer at pH 7.4, 150 mM NaCl and 1% Triton X-100, sonicated for 20 seconds and then centrifuged at 10,000 rpm for 10 min. The supernatants (homogenates) of each sample were maintained at −20 °C.

4.10. Lipid peroxidation measurements

Changes in lipid peroxide levels were determined using substances that react with thiobarbituric acid (TBARS), mainly

malondialdehyde (MDA), producing a pink-colored Schiff base as described previously.[48] Briefly, the homogenate (400 μg of protein) was incubated under agitation in a buffer containing 60 mM Tris–HCl (pH 7.4), 0.1 mM DPTA, 500 μl 12% TCA, and 0.73% TBA. The mixture was boiled for 2 h, cooled on ice and centrifuged at 10,000 rpm for 5 min. The absorbance of the supernatant was measured at 535 nm. The results were calculated using the molar extinction coefficient for malondialdehyde and expressed in nanomoles of TBARS per microgram of protein. Protein content was determined by Lowry's method.[46]

4.11. Mitochondrial potential measurement

To explore the effect of chalcones on mitochondrial membrane potential, the lipophilic cationic probe fluorochrome 5,58,6,68-tetraethylbenzimidazolcarbocyanine iodide (JC-1) was used. JC-1 is a green fluorescent monomer at depolarized membrane potential or a red fluorescent J-aggregate at hyperpolarized membrane potential. Cells were plated at 5×10^5 cells/well in 24-well dishes and incubated with the chalcones for 4 h. Afterward, JC-1 (10 μg/ml) was added and incubated for 20 min at 37 °C (5% CO_2), then cells were washed twice with PBS, resuspended in 500 μl of PBS. One hundred microliters was extracted and used to measure the fluorescence using a spectrofluorimeter (Perkin–Elmer LS55). JC-1 was excited at 488 nm, the red emission fluorescence was detected at 590 nm and the green fluorescence was detected at 527 nm. The mitochondrial potential was presented as a ratio of 590/527 fluorescence and compared with the control cells that were considered to have 100% fluorescence. An electron transport chain uncoupler (FCCP 1 μM) was used as a positive control.

4.12. Enzyme assays

Glutathione peroxidase (GPx) was assayed according to Flohé and Gunzler[49] using 150 μg of protein and NADPH oxidation was monitored by its decrease in absorbance at 340 nm ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$). Catalase activity was determined according to Aebi[50] using 60 μg of protein. In this assay, the disappearance of H_2O_2 was evaluated by measuring the decrease in absorbance at 240 nm (molar extinction coefficient: $\varepsilon = 40\ M^{-1}\ cm^{-1}$). Glutathione reductase was assayed according to Carlberg and Mannervick[51] and the NADPH oxidation, which resulted from GSSG reduction by GR, was determined by its decrease in absorbance at 340 nm ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$) using 150 μg of protein. Glutathione *S*-transferase was assayed according to Keen et al.[52] using 60 μg of protein. In this assay, GST induces the conjugation of GSH with CDNB. The conjugate was detected spectrophotometrically at 340 nm. The results were normalized by protein concentration and expressed as percentages of enzyme activity.

4.13. Glutathione measurement

Total glutathione (GSH + GSSG) was measured using the glutathione reductase method.[53] In this assay, homogenate (50 μg of protein) was transferred to a 96-well plate, to which a solution was added that contained 75 μM 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), 120 μM NADPH, 4 U/ml glutathione reductase (GR) and 10 mM EDTA in 200 mM phosphate buffer at pH 8.0. The absorbance was measured at 405 nm using a microwell systems reader. The concentrations of total glutathione (TG) and GSH were calculated using GSH as a standard and were normalized by protein concentration. The content of GSSG was calculated as follow: GSSG = (TG − GSH)/2. GSSG is equivalent to 2 GSH according to the following reaction: $GSSG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+$ in the presence of GR. The results were expressed as percentages.

4.14. ATP measurement

The intracellular ATP content was determined by a bioluminescence assay measuring the light output from the luciferin–luciferase reaction. First, 1×10^6 L1210 cells were incubated with the compounds (**R7** 23 μM, **R13** 37 μM, **R15** 36 μM) for 24 h. The cell extracts were obtained by homogenization with 1.25% trichloroacetic acid, then kept on ice for 30 min and neutralized with 1 M Tris–acetate at pH 7.5. After centrifugation, the supernatants were used for ATP quantification following the manufacturer's protocol. The results were normalized by the cell death percentage that occurs during 24 h of incubation.

4.15. Statistical analysis

The results were presented as means ± SD of triplicates from three-independent experiments. Statistical significance was assessed by ANOVA followed by Bonferroni's test, and $p < 0.05$ was taken as statistical significance.

Acknowledgments

This study was supported by grants from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and FAPESC (Fundação de Amparo à Pesquisa de Santa Catarina). This paper forms part of the Chemistry doctoral studies of Louise D. Chiaradia, who synthesize the compounds, and part of the Pharmacy master studies of Evelyn Winter. The group wishes to thank the Hospital Universitário (HU) for the use of its facilities and the Central de Análises (Departamento de Química, UFSC) for chemical analyses.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.09.025.

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